ON THE RÔLE OF THE OXIDATION IN THE METHYLATION OF GUANIDOACETIC ACID*

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(Received for publication, July 14, 1947)

There are two, at least, methyl transfer reactions promoted by liver slices *in vitro* (2). The fundamental distinction between them is that one is dependent on oxygen and the other is not.

Transmethylation reactions found to be independent of oxygen are methionine formation by the methylation of homocysteine, homocystine, or homocysteine thiolactone by either choline or betaine. They are not inhibited by oxidation inhibitors. Homogenized tissue and slices are equally effective without any modification of the reaction mixture.

Transmethylations found to be dependent on oxygen are the methylation of guanidoacetic acid to form creatine and of nicotinamide to N^1-methylnicotinamide (3). The methyl donor is methionine; choline and betaine are ineffective. Neither reaction in this category proceeds when the tissue (liver) is homogenized or finely minced and the reaction mixture consists only of methyl donor and acceptor.

After many unsuccessful experiments to gain further insight into the rôle of the oxidation in the methylation of guanidoacetic acid with rat liver slices we turned to the use of liver homogenates. We found that guanidoacetic acid is methylated by methionine in rat and guinea pig liver homogenates if sufficient adenosine triphosphate (ATP) is provided. The yields with rat liver homogenate were low and irregular. Guinea pig liver homogenates were better; they gave, on the average, more than twice the creatine obtained with rat liver slices (on the basis of dry weight of tissue used), and the results were consistent. The general picture of the reaction was the same as with rat liver slices. Accordingly we have confined ourselves in the study of the rôle of the oxidation in the methylation of guanidoacetic acid to homogenates of the livers of guinea pigs.

Methods

In the experiments with liver slices the animals used were adult white rats of both sexes which had been bred from the Wistar Institute strain.

* This work is a part of that done under contract with the Office of Naval Research, United States Navy Department. Presented at the meeting of the American Society of Biological Chemists, May 18–24, 1947 (1).

The homogenates were made from the livers of guinea pigs of both sexes, obtained commercially as needed.

The animals were killed by stunning and bled thoroughly. The technique, when liver slices were used, has been described in a previous communication (2).

For the preparation of the homogenates the liver was chilled in ice water immediately after removal, washed free of blood by ice-cold buffer solution, cut into small pieces, and then homogenized in the apparatus of Potter and Elvehjem (4) in a volume of ice-cold buffer solution equal to twice the weight of the tissue. The homogenization apparatus was jacketed in an ice and water bath. At the end of the homogenization the temperature of the homogenate had risen to 4°. In order to obtain a fine homogenate it was prepared in two stages, first in a tube in which the clearance of the rotor was 0.4 mm., followed, after straining through two layers of cheesecloth, by a second homogenization with a rotor clearance of 0.1 mm.

The buffer solution was made according to the recipe of Cohen and Hayano (5) at pH 7.4.

The reaction vessels were 20 ml. beakers. They were contained in a specially designed heat-regulated, gas-equilibrated reaction chamber.[1]

The final volume of the reaction mixtures (including that of the homogenate) was, in any one experiment, 3.0, 3.5, or 4.0 ml., whichever was convenient. The reaction mixtures were made up before the animal was killed and set away in an ice bath; the homogenate was pipetted into each as soon as it was prepared. The usual time from the killing of the animal to the beginning of the incubation at 38° was about 20 minutes. The contents of the reaction vessels came to the temperature of the bath in about 5 minutes. During this interval the oxygen or nitrogen was passed through in a vigorous stream, after which it was slowed down to a slight positive pressure which was maintained throughout the experimental period. From the time they were set in the water bath to the end of the experimental period the reaction vessels were rocked at a rate of 80 cycles per minute.

At the end of an experimental run the reaction was stopped by adding to each reaction vessel 2 drops of 1 N HCl. Water was then added to a desired volume, the pH adjusted to 5.0, and the vessels immersed in a boiling water bath for 5 minutes, after which they were cooled to room temperature and the contents filtered.

3 ml. aliquots were taken for analysis. 1 ml. of 0.4 N HCl was added to each; they were then autoclaved at 20 pounds pressure for ½ hour, cooled, the creatinine adsorbed on Lloyd's reagent, the clay washed twice with 3

[1] This apparatus was described briefly in a previous communication (2). A detailed description will be submitted for publication shortly.

ml. of 0.01 N HCl, and the creatinine assayed with alkaline picrate by the method previously described (6). Creatine standards were carried through the whole analytical procedure simultaneously with the experimental samples. The range of the standards and unknowns corresponded. A Klett-Summerson photoelectric colorimeter, with the green filter, was used.[2]

Reagents

The following were the substances used and their sources: guanidoacetic acid prepared by the method of Nencki and Sieber (11); N-phosphoguanidoacetic acid prepared by the method of Fawaz and Seraidarian (12); two preparations of L-methionine, one kindly donated by Professor W. C. Rose and one prepared by ourselves (2); D-methionine, also donated by Professor Rose; DL-methionine obtained from two commercial sources; L-methionine sulfoxide and L-methionine sulfone prepared by the methods of Toennies and Kolb (13, 14); L-dehydromethionine prepared by the method of Lavine (15); α-ketomethiol butyrate prepared by the method of Cahill and Rudolph (16); α-ketoglutaric acid prepared by the method of Schneider (17); fumaric acid purified by four recrystallizations from a commercial preparation; ATP prepared by the method of LePage (18); adenylic acid obtained from two commercial sources; cytochrome *c* prepared by the method of Keilin and Hartree (19); the oxidation inhibitors were commercial C.P. preparations.

[2] The alkaline picrate method is not specific for creatinine (7). Nevertheless we have used it for the following reasons. Determination of true creatine (as creatinine) by the difference in color with alkaline picrate before and after digestion with the specific bacterial preparation of Miller *et al.* (7, 8) is too cumbersome and time-consuming for experiments in which there are a large number of control and experimental solutions. And the number of variables which can be tested in any one experiment is greatly reduced because of the necessary duplication both of the analyses and of the number of samples of each experimental variable under test. Most of the non-creatinine chromogenic material (except glycocyamidine) is removed by adsorption on Lloyd's reagent and subsequent washing of the clay. In an earlier study (9) we found that deducting the color given by tissue alone plus that given by guanidoacetic acid alone gave, without prior bacterial digestion, values for creatine formed 90 $\pm$ 5 per cent of those obtained by difference before and after bacterial digestion. In this study we were interested only in the amount of creatine formed from guanidoacetic acid and not in absolute values. As will be seen below, with the method used (*i.e.* without bacterial digestion and deducting the tissue and guanidoacetic acid blank values) no creatine formation was noted when either guanidoacetic acid or methionine was withheld from the reaction mixture. The rate of creatine formation was greater the higher the initial concentration of either guanidoacetic acid or methionine. We consider, therefore, that the method used gave a true picture of creatine formation in the experiments described below. Handler and Bernheim (10) came to a similar conclusion.

When nitrogen was used for anaerobic experiments, the residual oxygen was removed by pasing it over red-hot copper filings in a muffle furnace.

Results

Table I is a summarized protocol of a typical experiment showing the formation of creatine from guanidoacetic acid in guinea pig liver homogenate. The figures show the necessity of methionine and a doubling of

TABLE I

Methylation of Guanidoacetic Acid to Creatine in Guinea Pig Liver Homogenate; Effect of Added ATP and of Methionine

Volume, 3 ml. 1 ml. of homogenate consisting of 1 part of liver and 2 parts of buffer. Initial concentrations of reactants, guanidoacetic acid $2.5 \times 10.^{-4}$ M, L-methionine 1.5×10^{-3} M, ATP (purity 93 per cent) 1.5×10^{-3} M. The pH of all solutions was adjusted to 7.4. The experiment was run for 1 hour at 38° under oxygen. The figures given are the averages of triplicates.

The gross "creatine" (Column 5) represents the total color developed in alkaline picrate compared with that of the creatine standards. The formed creatine (Column 6) represents (gross "creatine") − (tissue gross "creatine" + guanidoacetic acid gross "creatine"). In computing the figures in Column 7 it was assumed that the liver consists of 20 per cent solids. The dry weight of liver in each reaction vessel was on this basis 0.33 × 0.2 gm. = 66 mg. The slightly higher tissue blank with added ATP was due to color in the ATP preparation used.

Tissue	Guanidoacetic acid	Methionine	ATP	Gross "creatine" in filtrate	Formed creatine in filtrate	Formed creatine per gm. (dry weight) liver per hr.
(1)	(2)	(3)	(4)	(5)	(6)	(7)
				mg. per cent	*mg. per cent*	*micromoles*
+	−	−	−	0.70		
+	+	−	−	0.84	0	0
+	−	+	+	0.74		
+	+	+	−	1.09	0.25	2.3
+	+	−	+	0.88	0	0
+	+	+	+	1.44	0.56	5.2
−	+	−	−	0.14		

the yield by the addition of ATP with methionine. There was no methylation of guanidoacetic acid by the homogenate alone or when ATP was added without methionine.

The methylation of guanidoacetic acid by methionine when no ATP was added we have interpreted as due to ATP present in the homogenate or formed during the incubation. This interpretation is supported by the findings reported below, and by analogous findings on hippuric acid (20) and urea (21) synthesis in guinea pig liver homogenate. The latter reactions proceed without added ATP and are accelerated when it is added.

The augmenting effect of ATP varied in different homogenates. In eight other experiments the ratios of the amount of creatine formed with added ATP to the amount without ATP were 8.5, 6.6, 2.7, 2.2, 2.0, 1.9, 1.9, and 1.8.

The yield of creatine in guinea pig liver homogenate when ATP was added to the reaction mixture was, on the average, approximately double that given by rat liver slices. 5.2 micromoles per gm. (dry weight) of guinea pig liver per hour (Table I) was typical. In rat liver slices the yield on the same basis varied from 1.0 to 5.0 micromoles (9), with 2.5 to 3.0 micromoles as the median range.

The inferences from results such as those in Table I were strong that one function (if not the exclusive function) of the oxidation in the methylation of guanidoacetic acid by methionine in liver slices was the continuous provision of sufficient ATP, that the amount of the latter initially present in the slices was either insufficient or that it quickly disappeared, and that ATP produced in the slices as a concomitant of oxidations was available for the transmethylation reaction. It would support this interpretation if it were found in guinea pig liver homogenate that ATP produced during the incubation gave results similar to those by ATP added to the reaction mixture initially. Ochoa (22) showed that oxidation of α-ketoglutarate is obligatorily coupled with the esterification of inorganic phosphate and that in the process ATP is formed. Kalckar (23) found that the oxidation of malate or fumarate is associated with an intense phosphorylation; ATP formation was not demonstrated directly, but its intermediate formation could be inferred. Accordingly in a number of experiments adenylic acid plus either α-ketoglutarate or fumarate was added to the reaction mixture instead of ATP.

A typical set of results is given in Table II. They show that adenylic acid plus either α-ketoglutarate or fumarate was somewhat more effective than an initial addition of ATP, and as effective as ATP plus one of the acids. There was no additive effect of α-ketoglutarate and fumarate in the concentrations (0.01 M) used.

Results such as those in Table II strengthen the conclusion that ATP is required for the transmethylation and that one of the functions of oxygen both in slices and in homogenates is to support oxidations which provide ATP.

The interpretation we have put on the higher yield of creatine when either α-ketoglutarate or fumarate was added initially with ATP is that ATP diminishes in the homogenate during the incubation, whether by phosphatase action or by being used in reactions in addition to that of transmethylation. Hence when provision is made for its reconstitution by the addition and oxidation of such metabolites as α-ketoglutarate or fuma-

rate, a higher rate of transmethylations was maintained. This interpretation accounts, qualitatively, also for the somewhat higher yield of creatine with adenylic acid plus either α-ketoglutarate or fumarate than with ATP alone.

The addition of cytochrome *c* to the reaction mixture, over a concentration range of 10×10^{-6} to 4×10^{-6} M did not increase the yield of creatine.

TABLE II

Methylation of Guanidoacetic Acid to Creatine in Guinea Pig Liver Homogenate; Effect of ATP Formed during Reaction

Volume, 3.5 ml. 1 ml. of homogenate consisting of 1 part of liver and 2 parts of buffer. Initial concentrations of reactants, guanidoacetic acid 8×10^{-4} M, L-methionine 1×10^{-2} M, ATP (purity 93 per cent) 1.5×10^{-3} M, adenylic acid 1×10^{-3} M, α-ketoglutarate 1×10^{-2} M, fumarate 1×10^{-2} M. The pH of all solutions was adjusted to 7.4. The experiment was run for 1 hour at 38° under oxygen.

The figures given are the averages of triplicates.

The values in Columns 9 and 10 were computed on the same basis as the corresponding values in Table I.

Tissue	Guanidoacetic acid	Methionine	ATP	Adenylic acid	α-Ketoglutaric acid	Fumarate	Gross "creatine" in filtrate	Formed creatine in filtrate	Formed creatine per gm. (dry weight) liver per hr.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
							mg. per cent	*mg. per cent*	*micromoles*
+	−	+	−	+	+	+	0.72		
+	+	−	−	+	+	+	1.13	0	0
+	+	+	−	+	−	−	1.19	0.06	0.6
+	+	+	−	+	+	−	1.74	0.61	6.0
+	+	+	−	+	−	+	1.73	0.60	5.9
+	+	+	−	+	+	+	1.73	0.60	5.9
+	−	+	+	−	+	+	0.78		
+	+	−	+	−	+	+	1.19	0	0
+	+	+	+	−	−	−	1.70	0.51	5.0
+	+	+	+	−	+	−	1.83	0.64	6.3
+	+	+	+	−	−	+	1.86	0.67	6.6
+	+	+	+	−	+	+	1.80	0.61	6.0
−	+	−	−	−	−	−	0.41		

This was found in both rat and guinea pig liver homogenates, whether or not ATP, α-ketoglutarate, or fumarate was added. The concentration of cytochrome *c* is evidently not a limiting factor in the homogenates used.

We have explored, cursorily, the effect of the initial concentrations of the catalyst and of the major reactants on the rate of creatine formation. A more thorough study has been postponed until a purified enzyme preparation is available. Briefly the findings were as follows: The same amount of creatine was formed in 1 hour at 38° with 0.2 and 0.1 gm. of fresh liver

per ml. of reaction mixture; half that amount was obtained with 0.05 gm. and one-eighth with 0.025 gm. of liver per ml. of reaction mixture.

Tripling the initial concentration of guanidoacetic acid from 0.26×10^{-3} to 0.78×10^{-3} M increased the rate 50 per cent. Increasing the initial methionine concentration from 1×10^{-3} to 1×10^{-2} M also increased the rate 50 per cent.

The effects of increasing the initial concentrations of both guanidoacetic acid and of methionine were additive. The following is a typical result. With initial concentrations of guanidoacetic acid and of methionine 0.26×10^{-3} and 1×10^{-3} M, respectively, 2.0 micromoles of creatine were formed per gm. of dry weight of liver per hour; with 0.78×10^{-3} and 1×10^{-2} M guanidoacetic acid and methionine, respectively, the rate was 5.2 micromoles. Intermediate yields of creatine were obtained with intermediate concentrations of methyl acceptor and donor.

ATP added in initial concentrations of 0.003, 0.0015, and 0.00075 M gave the same yields of creatine, and twice that when no ATP was added.

The rate of creatine formation in guinea pig liver homogenate diminishes with time. Thus guanidoacetic acid and methionine gave with ATP alone in 1, 2, and 4 hours 5.9, 8.9, and 8.9 micromoles respectively per gm. (dry weight) of tissue; with ATP plus α-ketoglutarate the corresponding values were 6.3, 10.4, and 12.1 micromoles; and with adenylic acid plus α-ketoglutarate 7.3, 9.5, and 10.9 micromoles. In rat liver slices the rate continues nearly unslackened for 6 hours (9). Nearly all of the experiments with guinea pig liver homogenate were run for 1 hour, as more than half the amount formed in 4 hours was obtained in 1 hour and the complications of secondary reactions were lessened.

With rat liver slices, the addition of ATP to the reaction mixture neither accelerated the transmethylation nor did it relieve the inhibiting effect of anaerobiosis. In view of the results obtained with guinea pig liver homogenate the ineffectiveness of ATP with liver slices is to be ascribed to the inability of ATP to penetrate the liver cells.

Table III is a summary of experiments designed to ascertain the relative speeds with which isomers and oxidized derivatives of methionine methylate guanidoacetic acid in guinea pig liver homogenate. L-Methionine had twice the activity of its α-keto analogue and 5 times that of L-methionine sulfoxide. When the initial concentration of the sulfoxide was reduced 10-fold, to 1×10^{-3} M, it had no activity in the 1 hour period of the test. D-Methionine, L-methionine sulfone, and L-dehydromethionine were either only slightly active or inactive. DL-Methionine was slightly more active than L methionine. The activity of the α-keto and sulfoxide derivatives of methionine can be accounted for by inferring their prior conversion to L-methionine; less than 10 per cent conversion would suffice to account for the creatine they formed.

All of the compounds in Table III had been tested as possible methyl donors to guanidoacetic acid with rat liver slices by Handler and Bernheim (10) and by ourselves.[3] D-Methionine had about half the potency of L-methionine, and its effectiveness was nullified by 0.01 M benzoate, which inhibits D-amino acid oxidase (10). The α-keto anologue of methionine was as effective as L-methionine. Handler and Bernheim found the sulfoxide and sulfone of methionine to be ineffective. We found the sulfoxide and L-dehydromethionine to have 25 to 50 per cent the effectiveness of L-methionine and the sulfone to be ineffective.

TABLE III

Relative Speeds of Methylation of Guanidoacetic Acid to Creatine by Methionine Isomers and Derivatives

Volume, 3 ml. 1 ml. of homogenate consisting of 1 part of liver and 2 parts of buffer. Initial concentrations of reactants, guanidoacetic acid 1×10^{-3} M, ATP (purity 93 per cent) 1.5×10^{-3} M, α-ketoglutarate 1×10^{-2}. The experiment was run for 1 hour at 38° under oxygen.

The figures given are the averages of triplicates expressed as micromoles of creatine.

	Creatine formed
L-Methionine* (0.01 M)	5.5
D-Methionine† (0.01 ")	0.9
DL-Methionine (0.1 M)	6.3
L-Methionine sulfoxide (0.01 M)	1.2
" " (0.001 ")	0
L-Methionine disulfoxide (sulfone) (0.01 M)	−0.1
L-Dehydromethionine (0.01 M)	0.4
α-Ketomethiol butyrate (0.01 M)	2.9

* $[\alpha]_D^{25} = -7.5°$.
† $[\alpha]_D^{25} = +8.76$.

In vivo DL- is as effective as L-methionine for growth and lipotropic purposes, as is also the sulfoxide (24–26). The α-keto analogue can replace methionine for growth (16). D-Methionine is about as active as the L and D forms lipotropically. Methionine sulfone cannot replace methionine in the diet for growth (27).

All of the observations on the activity of the different derivatives of methionine (including our observation of the positive activity of methionine sulfoxide) in homogenates, liver slices, and *in vivo* are consistent. And they are in accord with the view that L-methionine is the active form of methionine in creatine formation, and that the activity of derivatives is proportional to their prior conversion to L-methionine.

[3] Unpublished observations.

All of the results exclude an oxidation product of methionine as the immediate methyl donor to guanidoacetic acid. They, therefore, also exclude oxidation of methionine as the basis of the dependence of the reaction in liver slices on oxygen, and the participation of ATP in such an oxidation. Another possible explanation of the dependence on oxygen and of the need of ATP was that the immediate methyl acceptor may be N-phosphoguanidoacetic acid, and that the phosphorylation is effected by ATP, analogous to that of creatine. In experiments to test this possibility with rat liver slices and rat and guinea pig liver homogenates it was found that N-phosphoguanidoacetic acid was in no case as effective as guanidoacetic acid

TABLE IV

Methylation of Guanidoacetic Acid to Creatine in Guinea Pig Liver Homogenate, Effect of Anaerobiosis

Volume, 3.5 ml. 1 ml. of homogenate consisting of 1 part of liver and 2 parts of buffer. Initial concentrations of reactants, guanidoacetic acid 1.5×10^{-3} M, DL-methionine 1×10^{-2} M, ATP (purity 93 per cent) 1.5×10^{-3} M, adenylic acid 1.3×10^{-3} M, α-ketoglutarate 1×10^{-2} M, fumarate 1×10^{-2} M. The pH of all the solutions was adjusted to 7.4. The experiment was run for 1 hour at 38° under either oxygen or nitrogen.

The figures given are the averages of triplicates expressed as micromoles of creatine formed per gm. (dry weight) of liver per hour.

Additional metabolite	ATP		Adenylic acid	
	Oxygen	Nitrogen	Oxygen	Nitrogen
	5.9	0.9	0.7	0.2
α-Ketoglutarate	7.1	2.2	6.6	1.8
Fumarate	6.6	1.2	7.2	0.2
α-Ketoglutarate + fumarate	7.3	2.3	7.2	1.8

with or without added ATP, the latter with or without α-ketoglutarate or fumarate. All of the results indicated that N-phosphoguanidoacetic acid was dephosphorylated before it was methylated to creatine.

If the dependence of the reaction in liver slices on oxygen were solely for the procurement of ATP, it would be expected that the initial addition of ATP to guinea pig liver homogenate would relieve the inhibitory effect of anaerobiosis to some extent. It would not be expected that the inhibition would be entirely relieved, because ATP is hydrolyzed and used in other ways during the incubation, and anaerobic dismutative oxidations will not reconstitute ATP as rapidly as when coupled with the more rapid and more extensive aerobic oxidations.

Table IV is a summary of results of experiments designed to test the foregoing hypothesis. When the reaction mixture contained only ATP in

addition to guanidoacetic acid and methionine, the inhibition of anaerobiosis was 85 per cent. This figure is not significantly different from that obtained with rat liver slices, which in a number of experiments ranged from 90 to 84 per cent.[3]

As was to be expected from previous experiments, addition of adenylic acid alone to the reaction mixture with homogenate gave very little creatine

TABLE V

Effect of Oxidation Inhibitors on Methylation of Guanidoacetic Acid by Methionine in Rat Liver Slices (without Added ATP or Fumarate) and in Guinea Pig Liver Homogenate (with Added ATP and Fumarate)

The experiments with rat liver slices were carried out in Krebs-Henseleit Ringer's solution (29) under 95 per cent oxygen and 5 per cent carbon dioxide at 38° for 4 hours. The initial concentrations of guanidoacetic acid and of DL-methionine were 1.5×10^{-4} M and 4.5×10^{-4} M respectively. The volume was 4 ml. The pH of all solutions was adjusted to 7.4. The dry weight of liver tissue in each reaction vessel ranged from 20 to 30 mg. The figures given are the averages of three experiments in which every experimental point and the controls were run in triplicate.

In the experiment with guinea pig liver homogenate the volume was 3 ml., 1 ml. of homogenate consisting of 1 part of liver and 2 parts of buffer (5). Initial concentrations of reactants, guanidoacetic acid 7.5×10^{-4} M, DL-methionine 1×10^{-2} M, fumarate 1×10^{-2} M, ATP (purity 93 per cent) 1.5×10^{-3} M. The pH of all solutions was adjusted to 7.4. These experiments were run for 1 hour at 38° under oxygen. The figures given are the averages of triplicates.

The figures represent the creatine formed, expressed as per cent of yield without inhibitor.

Inhibitor	Rat liver slices	Guinea pig liver homogenate
	100	100
Arsenate (0.001 M)	80	98
Arsenite (0.001 ")	11	74
Azide (0.001 M)	95	100
Fluoride (0.02 M)		58
Malonate (0.05 M)	100	126
Anaerobiosis	15	31

formation under oxygen and nearly none under nitrogen. When α-ketoglutarate and ATP were added to the reaction mixture with homogenate, the inhibitory effect of anaerobiosis was only 69 per cent; and with α-ketoglutarate and adenylic acid 73 per cent. Fumarate was less effective than α-ketoglutarate in counteracting anaerobiosis; added with ATP the inhibition was 78 per cent and with adenylic acid 97 per cent.

In general the same results were obtained with oxidation inhibitors as with anaerobiosis (Table V). Included in Table V are the effects of oxidation inhibitors on rat liver slices. The most effective among the inhibitors

tried were anaerobiosis and arsenite. The inhibition of the latter was largely, but not entirely, relieved in the homogenate by the addition of ATP and fumarate. The difference between liver slices and homogenate in the degree of inhibition by arsenite may be taken as evidence that the mechanism of arsenite inhibition in slices is inhibition of ATP formation as a consequence of inhibition of oxidations.

The inhibition by fluoride and acceleration by malonate (Table V) are at present unexplained.

The effect of malonate on the transmethylation appears to be complex, and there is a suggestion in the results with malonate (Table VI) that

TABLE VI

Effect of Malonate on Methylation of Guanidoacetic Acid to Creatine in Guinea Pig Liver Homogenate

Volume, 3 ml. 1 ml. of homogenate consisting of 1 part of liver and 2 parts of buffer. Initial concentrations of reactants, guanidoacetic acid 1.5×10^{-4} M, DL-methionine 1×10^{-2} M, α-ketoglutarate 1×10^{-2} M, fumarate 1×10^{-2} M, ATP (purity 93 per cent) 1.5×10^{-3} M, adenylic acid 1×10^{-3} M, malonate 5×10^{-2} M. The pH of all solutions was adjusted to 7.4. The experiment was run for 1 hour at 38° in oxygen. The figures given are the averages of triplicates, expressed as micromoles of creatine formed per gm. (dry weight) of liver per hour.

	Added metabolite	Without malonate	With malonate
Experiment A	ATP	7.05	3.1
	" + α-ketoglutarate	7.7	4.5
	Adenylic acid + α-ketoglutarate	7.5	3.5
Experiment B	ATP	4.1	2.4
	" + fumarate	5.9	7.5
	Adenylic acid + fumarate	5.1	7.6

oxygen may be required for purposes in addition to the provision of ATP. Guanidoacetic acid and methionine with additions of either ATP alone, ATP plus ketoglutarate, or adenylic acid plus α-ketoglutarate creatine formation was inhibited by malonate. When fumarate was substituted for α-ketoglutarate, malonate accelerated the transmethylation.

DISCUSSION

The results in Tables IV, V, and VI are open to two interpretations. One is that anaerobiosis, arsenite, fluoride, and malonate inhibit the formation of ATP and thus of creatine through inhibition of oxidations. The other interpretation is that oxygen is necessary to the transmethylation for a number of functions: one is the indirect one of providing ATP; another may be the production of a metabolite reactant necessary for the transmeth-

ylation; still a third may be that oxygen is necessary to support an oxidative step in the over-all transmethylation reaction. The second interpretation as it stands is little more than conjecture. It is prompted by the difference between the effects of α-ketoglutarate and fumarate, as is shown in Table VI.

We are at present engaged in a purification of the transmethylating enzyme system. When a satisfactory degree of purification is attained, the above alternative interpretations will be examined further. Until then, it seems unprofitable to discuss this aspect of the problem.

Regarding the precise function of ATP, the experimental results obtained exclude participation of ATP in either the oxidation of L-methionine or in the phosphorylation of guanidoacetic acid. Among the possibilities which remain are (1) formation of an intermediate by the condensation of L-methionine and guanidoacetic acid which is phosphorylyzed by ATP to creatine and a derivative of homocysteine, analogous to the cleavage of cystathionine (28); or (2) L-methionine is phosphorylated by ATP and the phosphorylated L-methionine is the immediate methyl donor to guanidoacetic acid. These possibilities are now under investigation.

The authors wish to acknowledge the assistance in this work of Miss I. Silberbach.

SUMMARY

1. The methylation of guanidoacetic acid by methionine proceeds in guinea pig liver homogenate. The rate, on the average, is double that in rat liver slices.

2. ATP is necessary for the transmethylation, and its formation is one of the reasons for the dependence of the transmethylation on oxidation reactions in liver slices and to a lesser extent in guinea pig liver homogenate.

3. The inhibition of the transmethylation by anaerobiosis or arsenite is partially relieved by ATP plus either α-ketoglutarate or fumarate.

4. Regarding the mechanism of the participation of ATP in the transmethylation the following two possibilities have been excluded: oxidation of L-methionine and phosphorylation of guanidoacetic acid.

5. Evidence is presented that the methyl donor is either L-methionine or a non-oxidative, possibly a phosphorylated, derivative.

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